PARTICULATE MATTER

Peer Review
Experimental Toxicology Division
NHEERL

Application of Emerging Technologies to Study In Vivo Alterations in Airway Intracellular Signaling Pathways Following Air Pollution Particle (PM) Exposure

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ABSTRACT

Earlier work attempting to link exposures to PM with pulmonary cellular toxicity has relied exclusively on *in vitro* data. Extrapolation of these data to the *in vivo* situation, with respect to specific injuries, locations, disease progression assessments, mode and mechanism of action, has been limited by the lack of appropriate, sensitive methodologies. Laser Capture Microdissection (LCM) technology has opened the way for exciting advancement in the area of *in vivo* toxicology. In these studies we have utilized LCM and protein array technologies to determine the *in vivo* molecular pathophysiology associated with emission source PM-induced site-specific lung injury. Conventional studies were first conducted using rats pretreated with antioxidants and then instilled with residual oil fly ash. Bronchoalveolar lavage techniques were employed to quantitate changes in the levels of known biomarkers of lung injury, and lung extracts were examined using immunohistochemical, biochemical, and molecular approaches to identify PM-induced alterations in intracellular signaling pathways and proinflammatory gene expression. These studies showed an inhibition of PM-induced pulmonary inflammation, cytotoxicity, ERK1/2 activation, and cytokine gene expression, thus demonstrating that oxidative stress is involved in these responses *in vivo*. LCM and protein array technologies validated the initial findings and indicated site-specific responses within airway epithelial cells. These studies allow for more definitive approaches to assess site-specific molecular pathology and mechanisms of injury in a variety of tissues and at more relevant exposures to environmental pollutants such as PM.

INTRODUCTION

Insight into the causal PM properties and pathological mechanisms by which PM mediates its adverse health effects is needed to provide biological plausibility to PM epidemiological associations. Elucidating the *in vivo* mechanisms of PM-induced pulmonary injury is challenging due to the physicochemical complexity and dynamic nature of ambient air PM as well as a lack of appropriate sensitive, specific and comprehensive cell and molecular technologies to investigate PM injury *in vivo*. Most of our understanding of how particles affect various pulmonary cell types is derived from *in vitro* studies. However, it is has been difficult to perform dose-response and mechanistic extrapolations to the *in vivo* situation from the existing *in vitro* PM mechanistic reports.

SPECIFIC OBJECTIVES:

1.To determine the *in vivo* pathophysiology associated with emission source PM-induced acute site specific lung injury.
2.To assess emerging technologies for the study of cardiopulmonary injury due to air pollution.

METHODS

Animals: Male, 60-90 day old, Sprague-Dawley rats were obtained from Charles River Laboratory, Raleigh, NC. Animals were maintained in a AAALAC approved animal facility and provided food and water *ad libitum*. The experimental protocols used in this study were in accordance with Institutional Animal Care and Use Committee guidelines.

ROFA Exposure: Residual oil fly ash (ROFA) particles were obtained from a power plant burning low sulfur #6 residual oil that have been extensively characterized. Animals were intra-tracheally instilled (IT) with saline or 0.5 mg/rat ROFA.

Assessment of Lung Injury: Rats were analyzed for biomarkers of acute lung injury at 24 h post-exposure by bronchoalveolar lavage (BAL). BAL fluid samples were analyzed to determine total protein, lactate dehydrogenase (LDH) release, and antioxidant activity using the Cobas Fara II. BAL fluid cell counts were determined using a Coulter counter. Differential cell counts were determined on BAL fluid cytospin preparations stained with LeukoStat.

Protein Analysis: Lung sections were immunostained to detect phospho-ERK1/2 using an antibody specific for phospho-ERK1/2. Lung tissue protein extracts were examined at 1h, 3h, and 24h post-exposure by Western and dot blot analyses using the same phospho-ERK1/2 specific antibody. Activity analysis was performed using the p44/42 MAP Kinase Assay Kit according to manufacturer's direction. Blots were developed using a peroxidase-conjugated goat anti-rabbit immunoglobulin G secondary antibody and the ECL+Plus detection kit.

Laser Capture Microdissection and Protein Microarray: OCT embedded whole lung samples taken at 6h and 24h post ROFA exposure were cut by cryostat into 10 µm sections, stained, and dehydrated. 3000 pulses/sample were extracted using 30 µl of extraction buffer. 3 nl of protein lysate was arrayed onto glass backed nitrocellulose slides using a GMS 417 microarrayer. Microarrays were developed using an automated slide stainer with a catalyzed signal amplification system.

Image Analysis: Microarrays were scanned on an Epson flatbed scanner and quantitation by optical density performed by ImageQuant Software

Statistics: Protein levels were normalized by dividing the median level of primary antibody expression by the medial level of actin expression. Data were presented as means ± SE and were analyzed using the GLM procedure of SAS. Differences between treatment means were determined using a t-test following a significant F-test.

Table 1
Bronchoalveolar lavage fluid biochemical and cellular changes in rats

Parameters, units	Treatments* Cage Control	Saline/Saline	DMTU/Saline	Saline/ROFA	DMTU/ROFA
BALF biochemistries					
LDH, mU/ml	26.3 ± 7.3	31.0 ± 2.2	22.7 ± 2.4	84.6 ± 3.8	41.0 ± 3.0[a,c]
Total protein, µg/ml	138.3 ± 19.9	172.0 ± 9.3	156.0 ± 6.7	365.8 ± 24.5[a]	279.6 ± 29.3[a]
Antioxidant activity, mM	0.07 ± 0.06	0.08 ± 0.01	0.26 ± 0.02	0.13 ± 0.03	0.32 ± 0.02[a,c]
BALF cell counts, x 10^3					
Cells/ml	53.1 ± 5.9	88.0 ± 13.0	79.0 ± 6.5	366.0 ± 86.8[a]	302.0 ± 49.7[a]
Macrophages, MO/ml	52.5 ± 5.6	86.0 ± 12.0	76.6 ± 6.5	92.0 ± 9.2	141.0 ± 17.5[a,c]
Neutrophils, PMN/ml	39.2 ± 2.0	2.00 ± 0.9	2.43 ± 0.7	271.0 ± 69.5[a]	116.0 ± 26.8[a,c]
Eosinophils, Eos/ml	0.20 ± 0.01	0.0 ± 0.0	0.2 ± 0.0	2.29 ± 0.6	3.22 ± 0.6[a]

*Treatment groups represent injection of either saline or DMTU (500 mg/kg) 30 min prior to intratracheal instillation of ROFA (0.5 mg/rat) or saline.
[a]Significantly different from Cage Control (p < .05).
[c]Significantly different from Saline/ROFA (p < .05).

Table 1. 24 h post exposure, rats exposed to intratracheally administered ROFA and intraperitoneal saline developed acute pulmonary injury characterized by significant increases in BALF concentrations of LDH and total protein. ROFA exposure also induced a neutrophilic and eosinophilic inflammatory response. Systemic administration of DMTU alone had no effect on the BALF indices, except for increased antioxidant activity. Administration of DMTU to ROFA instilled animals, however, significantly reduced LDH indices and the number of neutrophils and significantly increased the number of macrophages present in BALF.

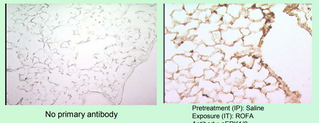

Figure 1. Antioxidant inhibition of the increase in pulmonary phospho-ERK1/2 levels following IT ROFA (500 µg) exposure. Lungs were formalin fixed, paraffin embedded, sectioned, and immunostained using a specific anti-phospho-ERK1/2 antibody. Shown are representative results from six separate animals at 3h post-exposure.

Results: Lung sections recovered from saline pretreated and IT-instilled rats were found to contain very low levels of pERK1/2. Increased diffuse as well as intense localized staining of pERK1/2 was clearly evident in the airway and alveolar regions within lung sections recovered from rats pretreated with saline and IT-instilled with ROFA. Pre-treatment of rats with DMTU 30 min prior to exposure was found to completely inhibit ROFA-induced increases in pERK1/2 levels.

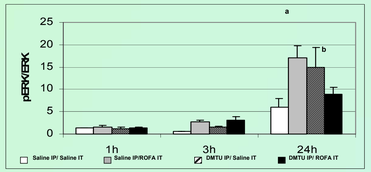

Figure 2. ROFA induced alterations in cellular signaling. Protein extracts were examined by dot blot analyses using a specific anti-p-ERK1/2 antibody. Western blotting confirmed antibody specificity. Values are means ± SE from three separate experiments. [a]Significant increase in the intensity of phospho-ERK1/2:ERK1/2 with ROFA IT compared to saline controls (p < 0.001). [b]Significant decrease in the intensity of phospho-ERK1/2:ERK1/2 with IP 30 min pre-treatment using DMTU prior to ROFA IT (p < 0.01).

RESULTS: Pretreatment of rats with saline followed by ROFA IT-instillations produced no increase in pERK1/2:ERK1/2 level at 1h post-exposure, a modest 2-fold increase in this ratio at 3h post-exposure, and a significant 6-fold increase of pERK1/2:ERK1/2 levels at 24h post-exposure. Pretreatment of rats with the antioxidant DMTU prior to ROFA exposure produced a significant 50 % inhibition in pulmonary pERK1/2:ERK1/2 levels at 24h post-exposure.

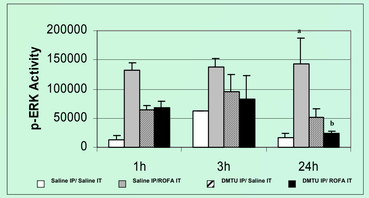

Figure 3. ROFA induced alterations in cell signaling activity. [a]Instillation with 500 µg ROFA resulted in a marked increase in phospho-ERK1/2 (p < 0.001). [b]Pre-treatment with 500 mg/kg DMTU resulted in a trend toward reduction in the activity of phospho-ERK1/2 at all time points, with the decrease at 24h being highly significant (p < 0.001).

Results: Early detection of ROFA induced alterations in ERK1,2 phosphorylation or activity was method dependent. Functional analyses demonstrated ERK1,2 activation at 1h post exposure as compared to elevated pERK1,2 at 3h post exposure, Figure 2. These results demonstrate that ROFA induced increases in pERK1,2 levels correspond with functional activation.

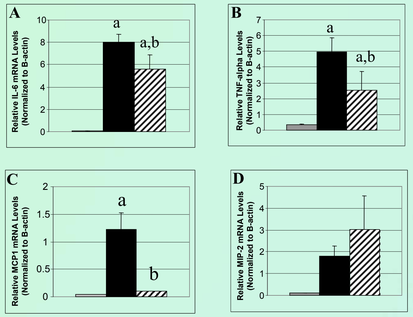

Figure 4. Antioxidant inhibition of ROFA-induced pulmonary proinflammatory gene induction. Rats were pre-treated with either saline (IP) or DMTU (500mg/kg SW, IP) thirty minutes prior to IT-instillation with saline or 0.5 mg/rat ROFA. RNA was recovered from lung tissues at 3h post-exposure for expression of: (A) IL-6 mRNA; (B) TNF-α mRNA; (C) MCP-1 mRNA; (D) MIP-2 mRNA; (E) IL-1 β mRNA; and at 24h post exposure expression of (F) matrilysin mRNA. Values are mean ± SE of six replicate samples.
DMTU, IP/ Saline, IT; Saline, IP/ROFA, IT; DMTU, IP/ ROFA, IT

Results: Pre-treatment of rats with the antioxidant DMTU significantly decreased ROFA induction of pro-inflammatory gene expression.

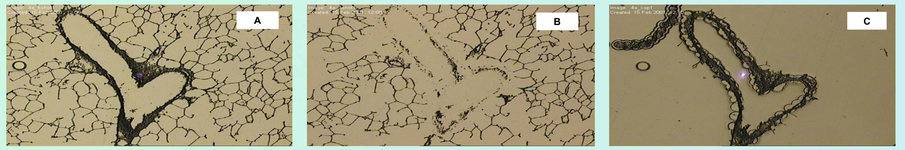

Figure 5. Visualization of LCM-procured cells. Laser capture microdissection (LCM) using the Arcturus Pixcell II was performed to selectively transfer the epithelial cells of a bronchus to the polymer film. A. Before LCM. B. Tissue after LCM; note the vacancy left by the removal of selected cells C. Epithelial cells transferred to cap surface.

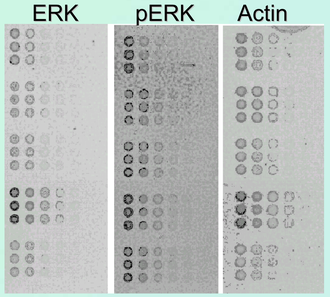

Figure 6. Protein array. After cell lysis, samples were arrayed with a pin and ring GMSE 470 microarrayer using a 375 micron pin onto nitrocellulose slides with a glass backing. Staining was carried out on an automated slide stainer using the Dako catalyzed signal amplification system. Samples were serial diluted and spotted in triplicate.

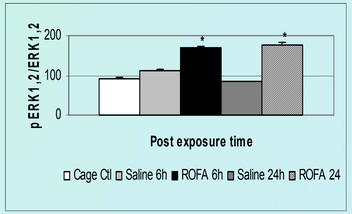

Figure 7. Densitometry analysis of protein array made from LCM procured pulmonary bronchi epithelial cells. Results were normalized to actin and displayed as a ratio of pERK:ERK. *Significant difference from cage control and saline (P < 0.001).

Results: No difference in the pERK1/2:ERK1/2 ratio was observed in cage control and saline treated animals at 6h or 24 h post-IT. In contrast, ROFA IT-instillation produced a significant 60% and 80% increase in pERK1/2:ERK1/2 levels at 6h and 24h post-exposure respectively

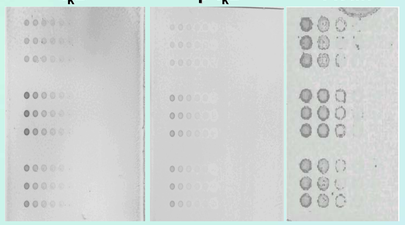

Figure 8. Protein array. After cell lysis, samples were arrayed with a pin and ring GMSE 470 microarrayer using a 375 micron pin onto nitrocellulose slides with a glass backing. Staining was carried out on an automated slide stainer using the Dako catalyzed signal amplification system. Samples were serial diluted and spotted in triplicate.

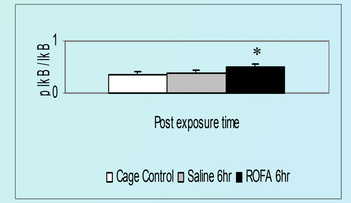

Figure 9. Densitometry analysis of protein array made from LCM procured pulmonary bronchi epithelial cells. Results were normalized to actin and displayed as a ratio of pIKB/IKB. *Significant difference from cage control and saline (P < 0.10).

Results: Cage control and saline IT-instilled rats did not demonstrate a significant increase in pI-κ_B-α :I-κ_B-α. However, a 30% increase in pI-κ_B-α :I-κ_B-α levels approaching significance was observed in microdissected airway cells recovered from ROFA exposed rats.

CONCLUSIONS

- Acute *in vivo* exposure to ROFA as a constituent of fine ambient air particulate pollution can induce pulmonary injury and inflammation and result in alterations in cell signaling and gene expression.
- Protein from airway epithelial cells can be extracted and analyzed to study signal transduction pathways using a microarray platform.
- These *in vivo* results validate similar *in vitro PM* mechanistic findings demonstrating for the first time the credibility of using LCM and protein array technologies to assess site-specific acute lung injury induced by specific air pollution particles.

IMPACT

- Provides coherence with *in vitro* PM mechanistic information thus allowing direct *in vitro* to *in vivo* extrapolation.
- Technologies to determine site specific pulmonary molecular pathology associated with exposure will provide biological plausibility to epidemiological studies by linking adverse health effects associated with ambient air PM exposure with specific emission sources.

FUTURE DIRECTIONS

- Improve our ability to identify PM affected and unaffected pulmonary and cardiovascular tissue regions for LCM microdissection (to document disease progression).
- Expand the array based approach for a more comprehensive assessment of altered cell signaling pathways.
- Link the pathophysiology associated with emission PM alterations in cell signaling pathways with the resonating cellular responses.

PUBLICATIONS

1. "Application of laser capture microdissection and protein microarray technologies in the molecular analysis of airway injury following pollution particle exposure." E. S. Roberts, L. Charboneau, V. Espina, L. A. Liotta, E. F. Petricoin, and K. L. Dreher. 2003 J. Tox. Environ. Health (In press).

2. "Oxidative stress mediates air pollution particle-induced acute lung injury and molecular pathology." E. S. Roberts, J. H. Richards, R. Jaskot, and K. L. Dreher. 2003. Inhal. Toxicol. (In press).

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